Organ Specificity in Hyperacute Rejection of Canine Heart and Kidney Allografts

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To clarify the organ specific nature of hyperacute rejection, 14 puppies were presensitized by multiple skin grafts and spleen cell injections prior to receiving either a heart or kidney allograft from the respective donors. Of this group, 7 received orthotopic heart allografts and 7 received kidney allografts. All heart allografts were rejected between 3 and 28 hours, and all kidneys between 0 and 24 hours as judged by cessation of urine flow from the ureterostomies. In contrast, all 11 animals in a recent series of heart allografts in non-sensitized puppies survived the operation, and rejected between 7 and 17 days. There was a significant correlation in both groups between preoperative cytotoxic antibody titer in the recipient serum and graft survival time. The preoperative titers were all above 1:1,024 but were greatly reduced within 2 hours after transplantation. At the time of rejection, antibody could be eluted from the rejected organs. In contrast to the kidneys, in which 2 of 7 grafts ceased to function immediately after revascularization, all hearts resumed beating and functioned well for at least several hours. At autopsy, the myocardium was pale and edematous and histologically polymorphonuclear leukocytes were prevalent in and around the small vessels and among myofibers. Both IgG and IgM antibody was detected in sarcolemma of the myocardium and to a lesser extent in the intima and adventitia of the small vessels by the fluorescent antibody technique. Biopsies of the rejected kidneys showed polymorphonuclear leukocyte infiltration, typical of hyperacute rejection. Marked fluorescence of IgG and IgM in the glomeruli and peritubular capillaries was observed. This study indicates that both organs rejected hyperacutely in our experimental model and participation of the preformed antibody in effecting this change was strongly suggested.

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WE HAVE PREVIOUSLY DESCRIBED an experimental model of orthotopic cardiac allotransplantation in which hyperacute rejection occurred consistently in the presence of high titer preformed cytotoxic antibody.[13] This phenomenon must be avoided in clinical heart transplantation, because of the drastic reaction that usually occurs as has been suggested by other investigators.[2,21]

The present study was designed to investigate the pathogenesis of hyperacute rejection of orthotopic cardiac allografts in comparison to that of renal allografts produced under similar experimental conditions. Special emphasis was placed on immunofluorescent and cytotoxic antibody studies.

Materials and Methods

Fourteen pairs of unrelated mongrel puppies of both sexes, weighing 3–5 kg, were prepared for presensitization using the most effective protocol derived from the previous investigation.[13] The initial sensitization was achieved with a 4 × 4 cm full-thickness skin graft transplanted from a potential heart or kidney donor followed by an intramuscular injection of spleen cells (1.0×10^9–5.4×10^9). The spleen cells were obtained by hemisplenectomy from the respective donor. Another series of skin and spleen cell injection was repeated alternately as the third and fourth procedures at approximately 2 week intervals. After completion of this protocol, seven puppies received orthotopic cardiac allografts and the remaining seven puppies received bilateral renal allografts from the respective donors.

The surgical procedure of orthotopic heart transplantation under profound hypothermia has been described in previous papers.[12,14] The heart grafts were transplanted by anatomosing both atria, the aorta, and the pulmonary artery under total circulatory arrest of the recipient animal. The electrocardiogram and femoral

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arterial pressure were monitored throughout the operative procedure and up to 2–4 hours after the operation.

Bilateral renal allotransplantation was performed as follows. The grafts were obtained by dissecting both kidneys with the segments of the abdominal aorta and vena cava in a block. The proximal stumps of these vessels were ligated, and heparinized lactated Ringer's solution (6–8 C) was irrigated from the distal stump of the aorta to flush out the blood. Side-to-end anastomosis with 5-0 Mersilene was made between the lower end of the host abdominal aorta and the distal stump of the graft aorta, and the lower end of the host inferior vena cava and the distal stump of the graft vena cava. The recipient was bilaterally nephrectomized immediately after transplantation. Condition of the grafts and urine flow from the ureters were monitored for 1 hour; then, if the grafts remained functional, the laparotomy was closed with two cutaneous ureterostomies in the lower abdomen. Since we did not encounter remarkable technical imperfections in this series, time of rejection was tentatively defined by the death of the animal in heart transplantation, and by cessation of the urine secretion from ureterostomies in kidney transplantation. Biopsy or autopsy was performed immediately thereafter for immunological and histological studies.

Sections for histologic study were fixed in 10% formalin and stained with hematoxylin-eosin.

Immunofluorescent Technique

Rabbit antidog-IgG and -IgM were prepared in our laboratory by the following methods. First, in order to increase the content of IgG and IgM in the canine serum, kidney allografting was performed on the dogs 7 to 9 days prior to exsanguination. Canine IgG and IgM, which were to be employed as antigens, were isolated from the serum by salting out twice with 50% saturated ammonium sulfate followed by gelfitration through a Sephadex G-200 column. The isolated IgG and IgM were purified by diethylaminoethyl (DEAE) cellulose column chromatography.[5] The purity of the IgG and IgM was determined immunoelectrophoretically against rabbit antidog serum (Cordis).

Then, approximately 1 mg of IgG or IgM in 1 ml volume was mixed with an equal part of complete Freund's adjuvant (Difco Laboratories, Detroit, Michigan). Five New Zealand white rabbits were immunized with the emulsion of IgG and five, with IgM by subcutaneous injection in foot pads and several parts of the back. The immunizations were repeated two more times at 3-week intervals in the same manner. Two or three weeks after the last immunization, the rabbits were exsanguinated.

To purify the sera, lipoproteins were precipitated with heparin and $MnCl_2$.[15] The lipoprotein-depleted serum

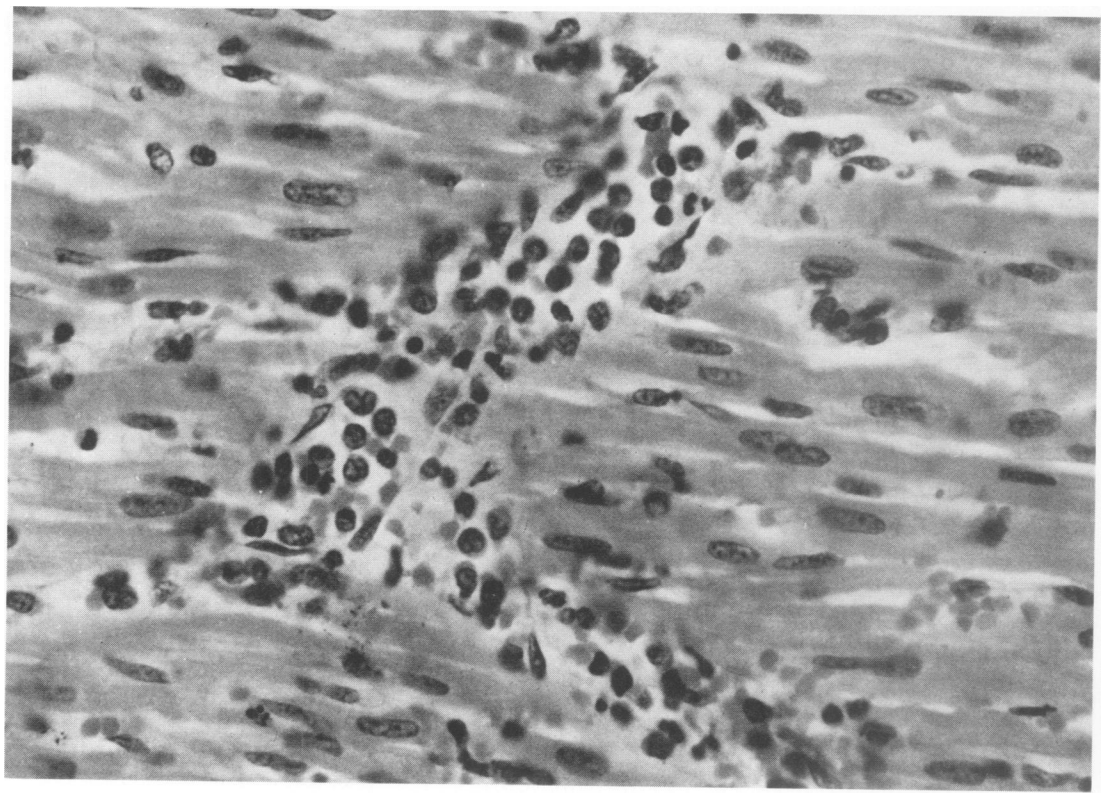

FIG. 1. Subendocardial myocardium from the heart rejected within 13 hours, demonstrating increased numbers of polymorphonuclear leukocytes in and around the small coronary vessels (Hematoxylin and eosin stain, ×130).

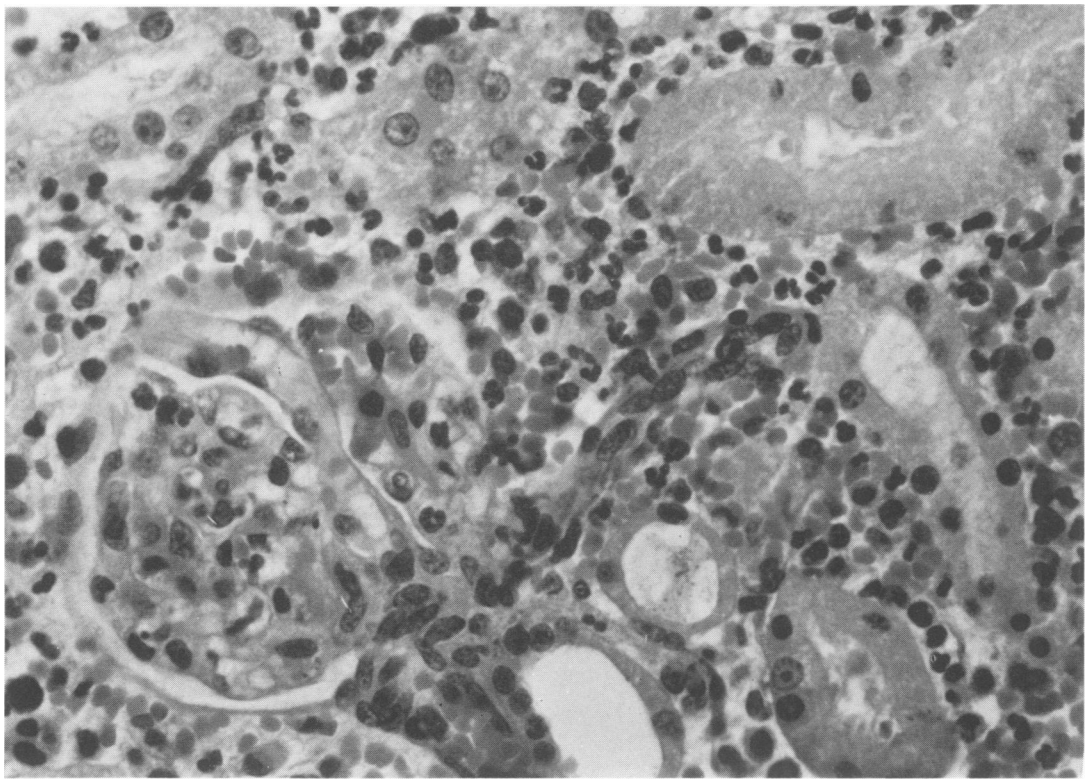

FIG. 2. Kidney graft rejected in hyperacute fashion within 7 hours, showing polymorphonuclear leukocyte accumulation in glomerular and peritubular capillaries (Hematoxylin and eosin stain, ×130).

was precipitated three times with ammonium sulfate at 33% saturation. The sediment was dissolved in phosphate buffered saline (PBS; pH 7.4) and, after removal of ammonium sulfate, the globulin was further purified by gelfiltration with Sephadex G-200 and DEAE cellulose chromatography. In order to reduce the cross reaction of IgG and IgM due to the common parts of the light chains, antidog-IgG and -IgM was absorbed with purified dog IgM and IgG respectively. Specificity of antidog-IgG and -IgM was examined by the Ouchterlony test and immunoelectrophoresis.

Purified anti-IgG and -IgM were labeled with fluorescein isothiocyanate (FITC) isomer 1 (crystallized BBL preparation) by the method of Kawamura.[8] Nonspecific staining activity of the labeled antibody was absorbed with acetone powder of mouse liver (Sylvania). The fraction with 1–2 molar ratio of fluorochrome to protein (F/P) was used for staining tissue sections.

Blocks of graft tissue obtained by biopsy or autopsy were frozen in n-hexane using a dry ice-acetone mixture or in liquid nitrogen and stored air-tight at less than

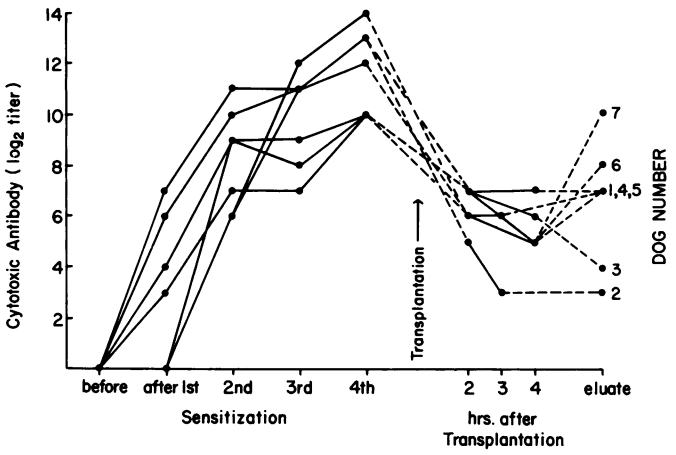

FIG. 3a. Changes in the titer of cytotoxic antibodies with heart or kidney (Fig. 3b.) allografts and leukoagglutinating antibodies with heart (Fig 3c.) or kidney (Fig. 3d.) allografts. Stepwise increase in titer through the sensitizations and abrupt drop after transplantation are clearly indicated in all figures. Eluate from the rejected grafts showed significantly high titer.

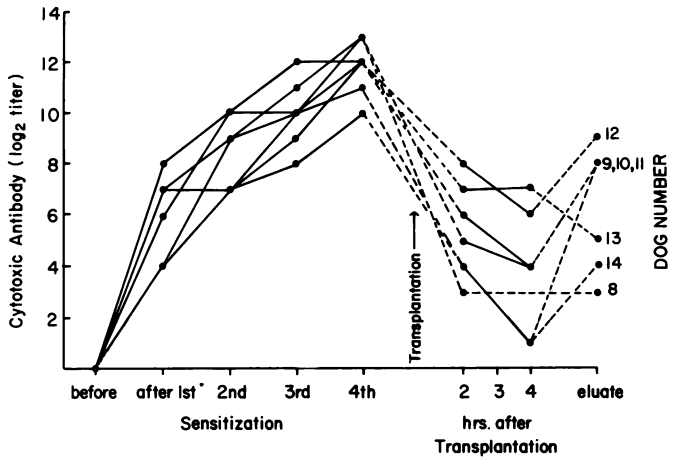

FIG. 3b.

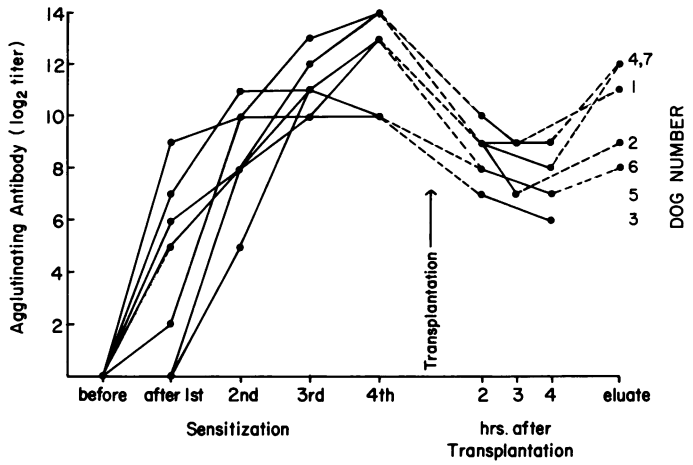

FIG. 3c.

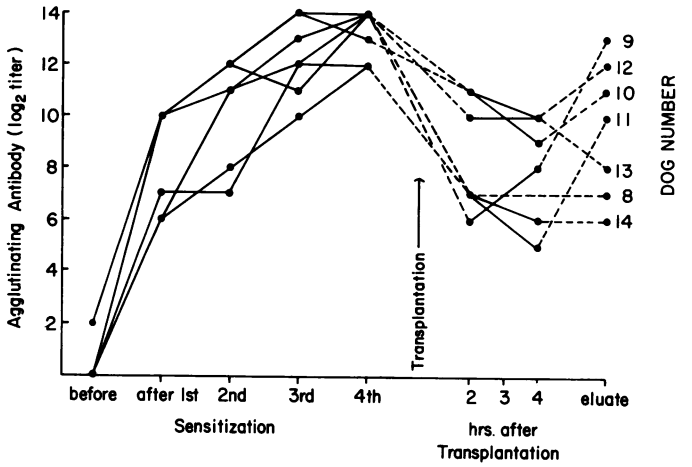

FIG. 3d.

−20 C. Cryostat sections 4 μ in thickness were airdried, washed in PBS for 10 minutes and used without fixing. Staining with the labeled anti-IgG or anti-IgM antibody was performed at 37 C for 30 minutes followed by washing with PBS for 2 hours and rinsing with distilled water. The sections were mounted in carbonate buffered glycerol and were examined on the same day. They were observed and photographed using an Olympus Universal Research Microscope Model VANOX with a FITC filter, an immersion dark field condenser, and a halogen lamp as a light source.

Cytotoxic and Leukoagglutinating Antibody Tests

Panels of test sera were prepared from peripheral blood of the potential recipients harvested just prior to each presensitization procedure, prior to transplantation, and 2 and 4 hours after transplantation. Eluates of the rejected grafts prepared by the method of Klassen and Milgrom[10] were included in the panels. The lymphocyte suspensions were prepared from heparinized donor blood using a Ficoll-Hypaque gradient isolation system.[18,20] Cytotoxic antibody tests were performed using the microtechniques described by Epstein[4] with minor modifications. One drop of lymphocyte suspension, one drop of rabbit complement (Hyland), and one drop of serial double-fold antiserum dilutions were mixed in a microtiter plate and incubated at 37 C for 60 minutes. After mixing with two drops of 0.25% solution of trypan blue in TC Hanks, the percentage of blue stained cells was counted. Cytotoxic antibody titer was defined as the highest dilution of antiserum which killed greater than 25% of the cells. The leukoagglutination test was performed using the technique of Dausset *et al.*[3]

Results

The 7 heart allografts were rejected between 3 and 28 hours and the 7 kidney allografts from immediate to 24 hours. In contrast, all 11 animals in a recent series of heart allografts[13] in non-sensitized puppies survived the operations and remained active and healthy until cardiac rejection occurred on from the 7th to the 17th day (mean, 11.7 days). One puppy of the present allograft group, which stood up, ate food and drank water the next morning, died at 28 hours of typical accelerated rejection. All hearts resumed beating and blood pressure was maintained sufficiently with or without minimal norepinephrine infusion. They awoke from anesthesia in a few hours, but in most instances remained lethargic. In two cases, regular heart beat was obtained spontaneously after cardiac massage for several minutes. However, the myocardium of the other animals appeared cyanotic, and repeated electric shocks were required to defibrillate the hearts. Generally, resuscitation of the

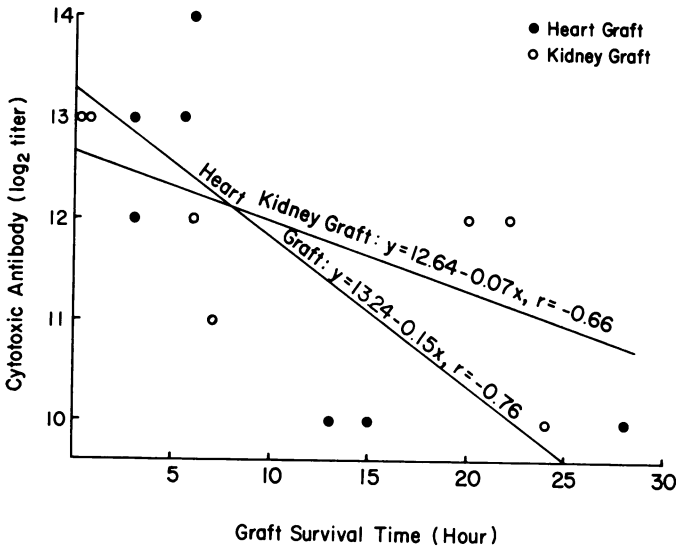

FIG. 4. Correlation between cytotoxic antibody titer and graft survival time.

TABLE 1. *Survival Time and Immunofluorescent Antibody Study on Heart Allografts in Presensitized Puppies*

Dog No.	Graft Survival Time (hours)	Immunofluorescence					
		IgM			IgG		
		Vessel	Sarcolemma	Interstitium	Vessel	Sarcolemma	Interstitium
1	3	++	++	−	++	++	−
2	3	+++	++	+	+++	+++	+
3	5½	++	++	+	++	++	+
4	6	++	+++	+	++	+++	+
5	13	++	++	+	++	+++	+
6	15	+	++	−	++	+++	+
7	28	++	+	+	+	++	+

Immunofluorescence was divided into 4 classes: +++, severe; ++, moderate; +, mild; and −, absent.

hearts in presensitized recipients was much more difficult than has been experienced in non-sensitized puppies. Two of seven kidney allografts ceased urine production shortly after revascularization; the remaining five allografts, between 6 and 24 hours after transplantation.

Histopathology

Autopsy revealed no technical imperfections to explain the early failure of the heart and kidney. Five hearts rejected within 13 hours appeared normal in size, although the right ventricle was usually distended. The myocardium was cyanotic, and contained scattered areas of subepicardial hemorrhage. This lesion could be attributed in part to surgical trauma, since subendocardial hemorrhage was rare. The lungs and abdominal organs revealed mild congestion. A distinct feature of these heart grafts was an increased number of polymorphonuclear leukocytes in some of the capillaries and perivascular areas. (Fig. 1). Some were arranged chain-like in a bloodless capillary, suggesting damage to the intima.

The hearts rejected at 15 and 28 hours demonstrated more definite pathology. The chest cavities contained an enlarged heart with slightly hemorrhagic exudate. The myocardium was dusky-red, rubbery and edematous with foci of hemorrhage. Histologic findings in the myocardium included mild mononuclear and polymorphonuclear leukocyte infiltration, interstitial edema, and hemorrhage.

Two kidney grafts, which were rejected almost immediately after restoration of blood flow, were characterized by mottling and cyanotic appearance, loss of turgor, cessation of urine excretion, and poor bleeding from the biopsy sites. The paired kidneys of a graft showed essentially the same attributes. Microscopically, there was an increased number of polymorphonuclear leukocytes within glomerular and peritubular capillaries (Fig. 2). The tubular epithelium revealed unremarkable changes.

The remaining five kidney grafts, rejected between 6 and 24 hours, showed severe congestion, swelling, and hemorrhage. After obtaining the open biopsy, four of them were left *in situ* until 24 to 48 hours after transplantation. All of them resembled grossly the ultimate rejection seen in unmodified renal allografts. Severe interstitial hemorrhage, congestion, probable tubular degeneration, and cellular infiltration, predominantly with polymorphonuclear cells were the uniform findings.

Cytotoxic and Leukoagglutinating Antibody Studies

As shown in Fig. 3, cytotoxic and leukoagglutinating antibody titers increased after each sensitizing procedure and all the puppies had titers above 1:1,024 at the time of grafting. Within 2 to 4 hours after grafting, the cyto-

TABLE 2. *Survival Time and Immunofluorescent Antibody Study on Kidney Allografts in Presensitized Puppies*

Dog No.	Graft Survival Time (hours)	Immunofluorescence					
		IgM			IgG		
		Vessel	Glomerulus	Tubulus	Vessel	Glomerulus	Tubulus
8	0	++	+++	+	++	+++	+
9	0	+	++	+	+	++	+
10	6	++	+++	+	++	+++	+
11	7	+	++	+	+	++	+
12	20	+	++	+	++	++	+
13	22	+	++	+	+	++	+
14	24	++	++	+	++	++	+

Immunofluorescence was divided into 4 classes: +++, severe; ++, moderate; +, mild; and −, absent.

toxic antibody titers were reduced to 1:128 or lower, and the leukoagglutinin titer to 1:1,028 or lower. Cytotoxic and leukoagglutinating antibody could be eluted in significantly high titer from the rejected heart muscle and kidney parenchyma (Fig. 3). There appeared to be a correlation in both groups between preoperative cytotoxic antibody titer in the recipient serum and graft survival time. The correlation coefficient was −0.76 in the group of heart grafts and −0.66 in the group of kidney grafts (Fig. 4).

Immunofluorescent Antibody Studies

The results of immunofluorescent studies in the rejected organ are summarized in Tables 1 and 2. There was intense fluorescence for IgG and IgM in the sarcolemma of the myocardium, but no specific fluorescence was found in the sarcoplasma. Therefore, microscopic observation of the transverse section revealed a honeycomb pattern (Fig. 5). Some capillaries in the myocardium showed strong fluorescence, although this finding was not consistent. Moderate deposition of IgG and less intensity of IgM were demonstrated in the perivascular connective tissue and intima of the small coronary arteries and veins. No fluorescence was detected in the large coronary vessels. In some cases, fluorescence positive mononuclear cells were seen sporadically in the vessels or myocardium.

In the rejected kidney grafts, there was marked fluorescence of IgG and IgM in the glomeruli and moderate fluorescence in the intima and adventitia of the small vessels (Fig. 6). Peritubular capillaries were also stained weakly with fluorescent antibodies. As a general rule, no clear difference was elucidated between the localization of IgG and IgM antibody. The specificity of the reaction was confirmed when fluorescence was blocked by pretreatment with unlabeled antidog-IgG and -IgM reagents.

Discussion

The method of presensitization of the recipient animal with the multiple skin grafts and spleen cell injections was very effective. All animals sensitized in this way revealed stepwise increases of donor specific lymphocytotoxic antibody titer with a final value of 1:1,024 or

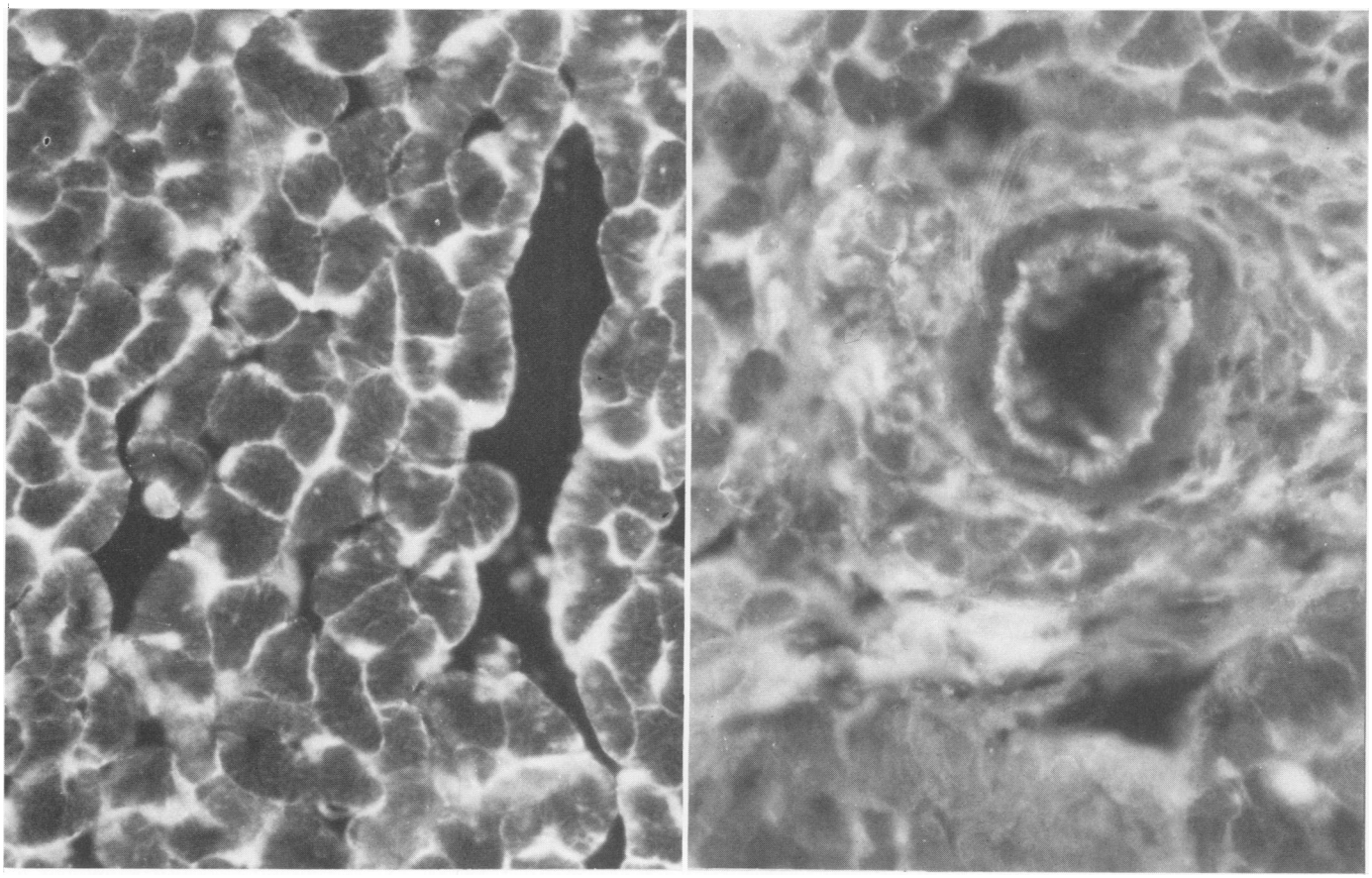

FIG. 5. The heart stained with fluorescent labeled anti-IgG reagent. (Left) Rejected after 6 hours. Note intense fluorescence in the sarcolemma of the myocardium (×130). (Right) Rejected after 13 hours. IgG accumulation around the small coronary vessels and in the sarcolemma (×130).

higher. Leukoagglutinating antibody titers also showed a parallel change. All of the kidney grafts and six of the seven heart grafts were rejected within 24 hours.

In our previous experiments,[13] we could not define a correlation between the titer of donor specific serum antibody in the recipient and the survival time of the grafted heart. Several other investigators working in the area of hyperacute rejection also failed to observe any correlation.[1,7,16] However, the present data suggest that there is a good correlation between the survival time of the heart or kidney grafts and the cytotoxic antibody titer in the pretransplant recipient serum. This discrepancy may be related to improvements in presensitization procedure and technical differences in cytotoxic antibody test. Mullerworth *et al.*[17] found a similar correlation between rejection time of the heterotopic cardiac allografts and the serum antibody titer in the dog. The rapid decline in serum antibody titer following transplantation and the presence of antibody in the eluate of rejected organs again support our previous assumption that antibody is absorbed selectively by the graft tissue.

Using immunofluorescent techniques, several investigators[6,17,19] detected IgG on the sarcolemma of cardiac allografts in various phases of rejection including the hyperacute, accelerated and acute rejection. No IgG was detected in sarcoplasma, although Heron[7] indicated the intracellular deposition of γ-globulin in degenerated myofibers. This study clearly demonstrated sarcolemmal IgG and IgM in the hyperacutely rejected myocardium. We could not detect fluorescence in the sarcoplasma. Because of the limitation of the method, we could not find the correlation between the intensity or localization of the fluorescence on the sarcolemma and the time of rejection. Existence of fluorescence in the wall of small vessels, as observed in this study, was also recognized by Mullerworth *et al.*[17] However, in the present study large coronary vessels showed no fluorescence and this observation was in contrast to the report by Heron[7] in heterotopic rabbit heart allografts; γ-globu-

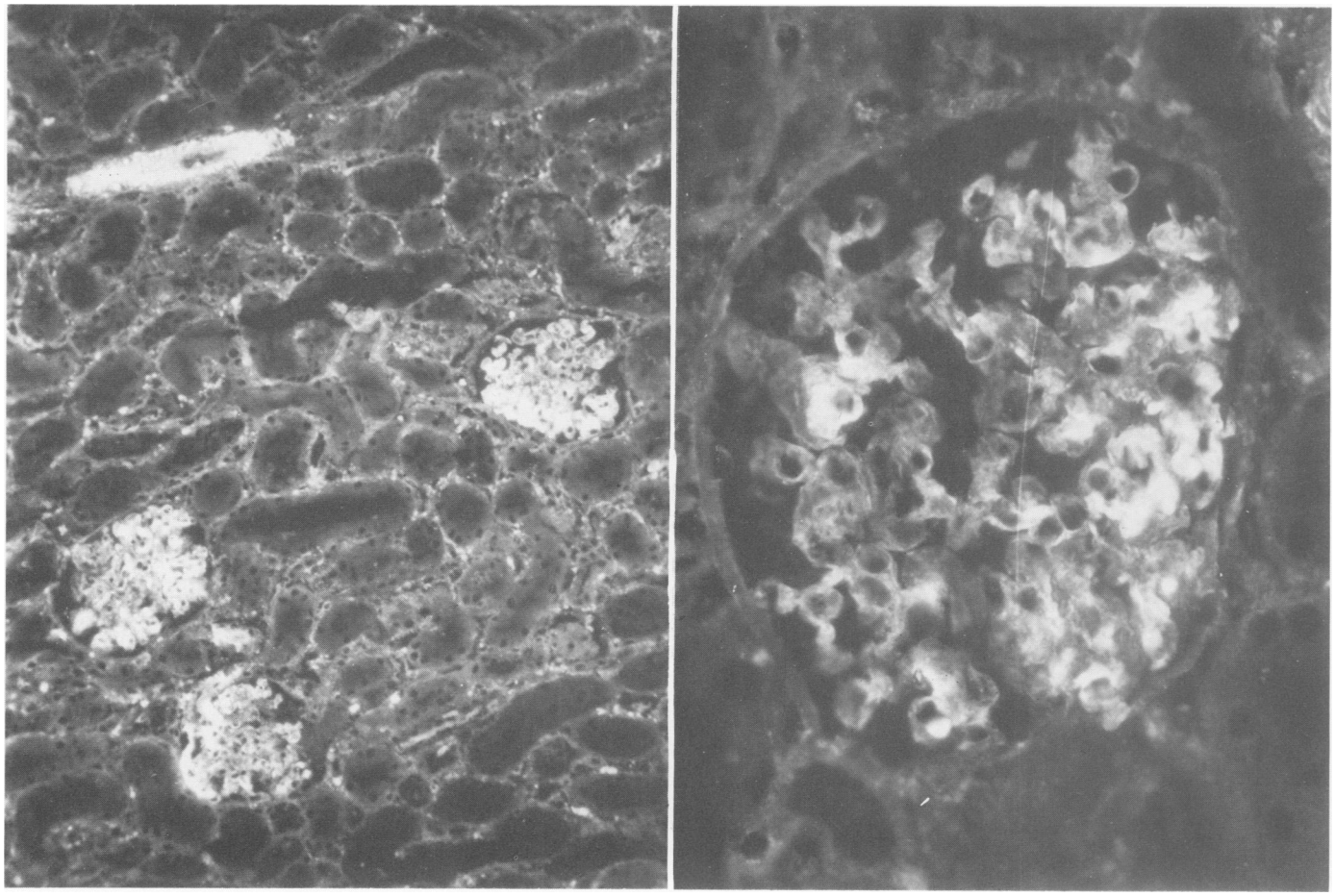

FIG. 6. The kidney stained with fluorescent labeled anti-IgG reagent. (Left) Rejected after 6 hours. Marked fluorescence of IgG in the glomeruli and moderate fluorescence in the intima and adventitia of the small vessel (×130). (Right) Rejected after 6 hours. Irregular accumulation of IgG in the glomerulus (×130).

lin was bound to the intima, and incorporated into the media and adventitia of some large vessels. In the orthotopic heart allografts, changes observed at the time of cessation of function are milder than those observed in the heterotopic allografts, in which coronary flow of the graft is maintained by host cardiac performance beyond the stage of functional end point. Although fluorescent positive mononuclear cells were seen, their character and function were not pursued.

In the rejected kidney grafts, fluorescence of IgG and IgM observed in the glomeruli was most marked in the glomerular capillaries. The intensity was variable among the glomeruli both in the same section and between the areas in the same glomerulus. Peritubular fluorescence was weaker than that of the glomeruli. These findings suggest that dysfunction of the kidney in the course of the hyperacute rejection process occurs due to the deterioration of glomerular function. Comparing the hyperacute rejection of heart and kidney grafts, there was a notable difference in the time of rejection. Two of seven kidneys were rejected almost immediately after restoration of blood flow. Such vigorous immediate rejection of the heart has not been observed in our experience. Williams *et al.*[21] and Kissmeyer-Nielsen *et al.*[9] reported cases of human renal allotransplantation which were rejected hyperacutely within 1 hour. MacDonald *et al.*[16] and Kobayashi *et al.*[11] have reported immediate rejection after revascularization of canine renal allotransplantation. No instance of hyperacute rejection in clinical heart transplantation has been reported, although an accelerated type of rejection has been described.[2,21] Although we do not have definite knowledge to explain these differences, we can assume that the vasculature of the kidney is more sensitive to immunologic insult than is the heart, and that the candidates for renal transplantation have more opportunity to presensitize through repeated hemodialysis.

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